Immunology, 1971, **20**, 67.

Phagocytosis of Murine Lymphoma Cells by Macrophages

I. FACTORS AFFECTING *IN VITRO* PHAGOCYTOSIS

R. EVANS

Chester Beatty Research Institute, Belmont, Sutton, Surrey

(*Received 6th February* 1970)

Summary. Factors affecting *in vitro* phagocytosis of a DBA/2 lymphoma, L5178Y, were investigated. Peritoneal macrophages from mice injected with thioglycollate medium were found to take up more lymphoma cells than those from normal mice. The number of cells phagocytosed by normal macrophages was found to be related to the concentration of antiserum employed and the number of lymphoma cells added to each macrophage slip culture. Treatment of macrophages or lymphoma cells with either trypsin or neuraminidase prior to the opsonic test greatly increased the number of cells ingested and also the number of macrophages containing ingested lymphoma cells. The relevance of the results from this opsonic test system to *in vivo* findings is discussed.

INTRODUCTION

Peritoneal macrophages have been shown to ingest a variety of tumour cells *in vitro* in the presence of allogeneic antiserum (Bennett, Old and Boyse, 1963, 1964). These authors demonstrated that phagocytosis was a measure of the opsonizing ability of antiserum, and some of the factors which affected this process were described. For example, it was found that tumour specific antiserum was the best source of opsonin, that phagocytosis of tumour cells was related to the concentration of antiserum employed, and that the use of peritoneal macrophages from normal mice, immunized mice or from normal mice previously injected with starch, affected the ingestion of tumour cells. The present report extends these observations which make use of a DBA/2 lymphoma, L5178Y, described by Fischer (1958), and antiserum from allogeneic CB57B1 mice immunized with L5178Y cells, which differ at the H-2 locus.

MATERIALS AND METHODS

Mice

C57B1 mice were used as a source of peritoneal macrophages and for the production of antiserum against lymphoma cells.

Cells

(i) DBA/2 L5178Y lymphoma cells were grown in the peritoneal cavity of syngeneic DBA/2 female mice and harvested 7 days after injecting mice with 5×10^5 cells. The cells were washed $\times 3$ in Fischer's medium free of serum and resuspended in Fischer's medium supplemented with 10 per cent heat-inactivated foetal bovine serum (GM) at concentrations referred to below.

(ii) Peritoneal macrophages were obtained from normal C57Bl mice or from mice injected i.p. with 1 ml sodium thioglycollate (Difco: Fluid Thioglycollate medium, dehydrated) 6 days previously. Macrophages were harvested after injecting mice with 3 ml of Fischer's medium, and then withdrawing the suspension with a syringe after gentle massage of the abdomen. Approximately 10^5 macrophages in 0·2 ml of GM were pipetted on to 16 mm circular cover slips in 5 cm plastic petri dishes, four slips per dish. Slip cultures were left at room temperature for 15 minutes then washed ×3 by immersion in phosphate buffered saline (PBS). 4 ml of GM were then added to each dish and cultures were incubated at 37° in an atmosphere of 5 per cent carbon dioxide in air. For most experiments macrophages from normal mice were used and allowed 24 hours to spread out on the glass.

Antiserum

Primary response allogeneic C57Bl serum directed against DBA/2 L5178Y cells was collected 10–14 days after i.p. injection of 4×10^6 L5178Y cells, this time being optimal for peak opsonic activity (Evans, 1971). Normal and immune sera were heated at 56° for 1 hour and dialysed overnight against Fischer's medium, and were either used immediately or stored in 1 ml volumes at −30°.

Experimental procedures

(a) *Opsonic test.* The method used was a modification of that described by Bennett *et al.* (1963). Serial five-fold dilutions (0·4 ml) of normal and immune serum, initially diluted 1/2 in Fischer's medium, were prepared in plastic tubes. Lymphoma cells in 0·1 ml volumes were added to each serum dilution at concentrations given in the text. After mixing well, 0·2 ml volumes of each dilution with the suspended lymphoma cells were pipetted on to each of two macrophage slip cultures, which had been removed from their original 5 cm dishes, blotted on their undersurface and placed in dry dishes, two slips per dish. After all dilutions had been dispensed in this way, the slips were incubated at 37° for 1·5–2 hours. This was followed by their immersion in PBS, which was pipetted over the surface of the macrophages to remove L5178Y cells not phagocytosed. Slips were then fixed in absolute methanol, and stained in Giemsa. The degree of phagocytosis was assessed by counting the number of macrophages with ingested lymphoma cells in fifty microscopic fields, using a ×10 objective and ×8 eyepieces. The percentage of macrophages with ingested lymphoma cells was calculated in relation to the total macrophage count. The dilution end point denoting positive phagocytosis was taken from the mean value of many experiments as that showing at least 1 per cent of the macrophages with ingested lymphoma cells. Normal serum, or controls without serum, generally showed no opsonic ability.

(b) *Enzymic treatment of cells.* (i) Neuraminidase (Behringwerke, Germany) was isolated from the filtrate of *Vibrio cholerae* cultures with a quoted strength of 500 units/ml of acetate buffer. To ascertain if treatment of lymphoma cells with the enzyme affected their subsequent phagocytosis, the following procedure was used. Dilutions of neuraminidase containing 250, 100, 50, 10 and 1 units per ml of Fischer's medium (to which 1 N NaOH was added to bring the pH up to 7·0) were mixed with 10^7 lymphoma cells/ml and incubated for 30 minutes at 37°. The cells were then centrifuged, washed ×3 in Fischer's medium and finally resuspended in Fischer's medium. They were then mixed with normal or immune serum, diluted 1/50, in preparation for the opsonic test.

(ii) Trypsin solution (2·5 per cent trypsin in normal saline, Flow Laboratories) was diluted to 1, 0·5, 0·25 and 0·1 per cent solutions, pH 7·4, and the same concentration of

lymphoma cells as in (i) was added. The suspensions were incubated for 30 minutes followed by the same procedure for washing, resuspending and finally mixing with 1/50 serum for the opsonic test. Approximately 5×10^5 L5178Y cells from (i) and (ii) were added to each macrophage slip culture.

Neither type of enzymic treatment affected the viability of the population, as measured by exclusion of trypan blue.

RESULTS

PHAGOCYTOSIS BY MACROPHAGES FROM NORMAL MICE AND FROM THOSE INJECTED WITH THIOGLYCOLLATE

Intra-peritoneal injection of thioglycollate greatly increases the number of macrophages found in the cavity 6 days later. Compared with normal macrophages, they have a greater volume of cytoplasm, which is usually finely vacuolated, and on contact with glass the cells spread out very rapidly. Because normal macrophages take much longer to spread, both types were allowed 24 hours to spread on cover slips, and then compared for their capacity to ingest L5178Y cells in the presence of anti-lymphoma serum.

TABLE 1

PHAGOCYTOSIS OF L5178Y CELLS BY MACROPHAGES FROM NORMAL AND THIOGLYCOLLATE-STIMULATED MICE

Macrophages from:	Per cent macrophages* with ingested L5178Y cells at each serum dilution				
	1/10	1/50	1/250	1/1250	1/6250
Normal mice	65	16	6	1	0
Thioglycollate-stimulated mice	70	20	7	2	0

* Mean of six experiments, using two to four slips per dilution.

Table 1 shows the dilution of antiserum together with the mean percentage of macrophages with one or more ingested lymphoma cells. While there was no difference in the dilution end points, the percentage of thioglycollate-stimulated macrophages containing ingested lymphoma cells was in general higher, and their cytoplasm frequently contained up to five lymphoma cells (Fig. 1). Normal macrophages rarely contained more than two lymphoma cells. Although thioglycollate-stimulated macrophages were well spread out within 1 hour of seeding on to glass, their use at this time did not increase the sensitivity of the test.

THE INTER-RELATIONSHIP OF FACTORS AFFECTING PHAGOCYTOSIS

The relationship between the concentration of immune serum and the number of cells ingested was examined, together with the influence of the size of the inoculum on the number of cells phagocytosed. Several series of antiserum dilutions were prepared, and each series was mixed with a different number of L5178Y cells to give a final ratio of 100 to 0·01 L5178Y cells : 1 macrophage when the dilutions were added to the slip cultures (these ratios represent inoculation of 10^7–10^3 L5178Y cells on to each slip). The slips were

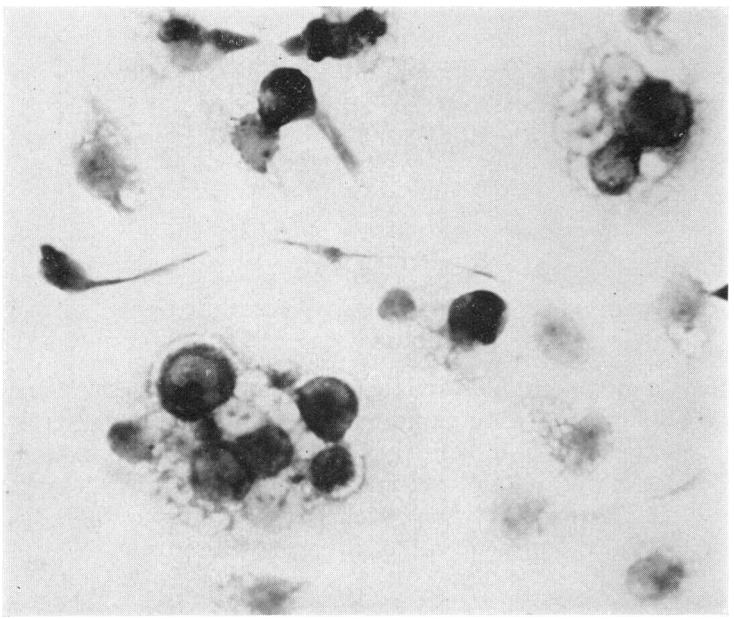

FIG. 1. *In vitro* phagocytosis of lymphoma cells by macrophages from mice injected with thioglycollate medium. Cytoplasm of macrophages is finely vacuolated. Giemsa, ×250.

then incubated for 2 hours, followed by examination for the degree of phagocytosis. In Fig. 2 the ordinate shows the logarithm of the number of lymphoma cells ingested, the abscissa showing the serum dilution, and each curve relates to the dose of cells added to each slip culture (cell inocula of 10^3–10^6 only are shown). When either 10^5 or 10^6 cells were added to slip cultures, the degree of phagocytosis was similar, as shown by the upper curves. The dilution end point was 1/1250, though occasionally phagocytosis at a dilution of 1/6250 was seen. Using smaller inocula of 10^3 and 10^4 cells, proportionately fewer cells were phagocytosed as the serum became more dilute. Thus, at a dilution of 1/250, the serum evidently contained sufficient opsonic activity to promote phagocytosis of at least 10^4 cells (upper curves), yet when the inocula contained only 10^3 or 10^4 cells very few were phagocytosed at this dilution. When the inocula contained more than 10^6 cells, massive clumping and adhesion of lymphoma cells to the macrophages took place, and this frequently obscured phagocytosis. When the clumps were removed by vigorous washing little phagocytosis was seen at 1/10 or 1/50 dilutions. Beyond these dilutions the clumping effect diminished, though phagocytosis was still somewhat masked by adhesion. In subsequent experiments, such large inocula were avoided and results indicated that doses of 5×10^4 to 5×10^5 cells per slip (= ratios of 0·5–5 L5178Y cells : 1 macrophage) produced maximal phagocytosis, and that the assay system was most sensitive in terms of dilution end point within this range.

Enzymic treatment of cells

(a) *Neuraminidase treatment.* Table 2 summarizes the results of incubating L5178Y cells in 1–250 units of neuraminidase prior to opsonization with antiserum diluted to 1/50. It is seen that compared with untreated cells, there was no difference after treatment of L5178Y cells with 1 and 10 units per ml. However, 50–250 units markedly increased the number of L5178Y cells phagocytosed. As many as eight L5178Y cells were seen within

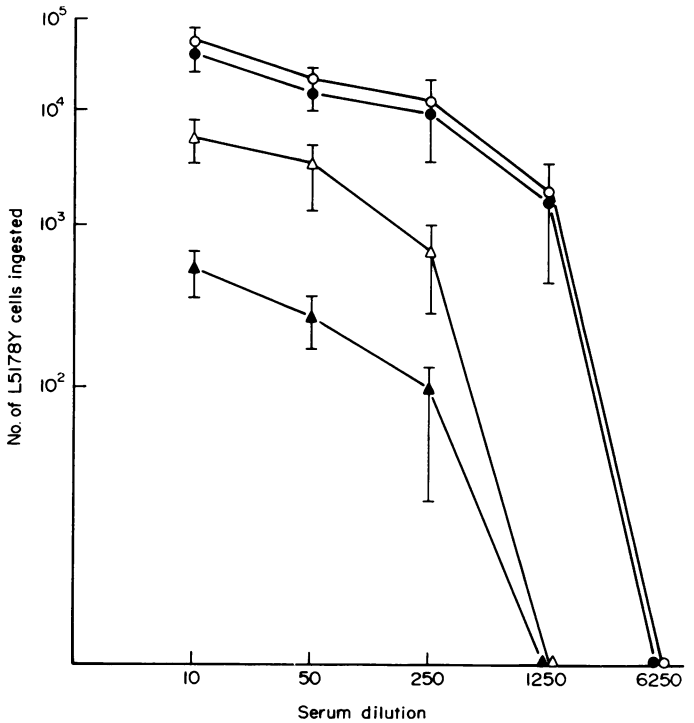

FIG. 2. The inter-relationship of the number of cells ingested by normal macrophages *in vitro*, the concentration of anti-L5178Y serum, and the number of cells in the initial inoculum. Cell inocula: ○, 10^6; ●, 10^5; △, 10^4; ▲ 10^3.

a single macrophage. The number of macrophages with ingested lymphoma cells was also increased. It was evident that 50 units/ml were as effective as 250 units/ml. To find out if the sensitivity of the test in terms of the dilution end point could be increased, lymphoma cells were incubated with 50 units neuraminidase per ml, followed by washing and then resuspension in serial five-fold dilutions of antiserum. Four macrophage slip cultures were used for each dilution containing 5×10^5 lymphoma cells for each culture. The results indicated that neuraminidase treatment increased the dilution end point from 1/1250 for untreated lymphoma cells (mean percentage of macrophages with ingested lymphoma cells at this dilution was 1·6) to 1/6250 for enzyme-treated L5178Y cells (mean percentage of

TABLE 2

THE EFFECT OF NEURAMINIDASE TREATMENT ON PHAGOCYTOSIS* OF L5178Y CELLS BY NORMAL MACROPHAGES

L5178Y cells incubated with:	Per cent macrophages with ingested L5178Y cells
Fischer's medium only	45–65; maximum of three L5178Y cells per macrophage
1 unit neuraminidase	50–65; maximum of three L5178Y cells per macrophage
10 units neuraminidase	Similar
50 units neuraminidase	65–85; maximum of eight L5178Y cells per macrophage
100 units neuraminidase	Similar
250 units neuraminidase	Similar

* Anti-L5178Y serum diluted 1/50.

TABLE 3

PHAGOCYTOSIS OF L5178Y CELLS FOLLOWING NEURAMINIDASE* TREATMENT OF NORMAL MACROPHAGES, LYMPHOMA CELLS OR BOTH CELL TYPES

Cells	Per cent macrophages with ingested L5178Y cells
Treated macrophages + untreated L5178Y cells	70–80; up to eight L5178Y cells per macrophage
Treated macrophage + treated L5178Y cells	Similar
Untreated macrophages + untreated L5178Y cells	45–65; up to three L5178Y cells per macrophage
Untreated macrophages + treated L5178Y cells	75–85; up to eight L5178Y cells per macrophage
Above combinations + normal serum	No phagocytosis

* Cells incubated with 50 units neuraminidase per ml.

macrophages with ingested lymphoma cells at 1/1250 was 4·2 and at 1/6250 it was 1·1). Normal serum did not opsonize treated or untreated lymphoma cells.

To test whether increased phagocytosis occurred if macrophages only were treated with neuraminidase, normal macrophage slip cultures were incubated with 50 units of neuraminidase. They were washed and subsequently inoculated with 5×10^5 untreated or neuraminidase-treated lymphoma cells suspended in normal or immune serum diluted to 1/50. These were compared with untreated macrophage cultures inoculated with untreated or enzyme-treated lymphoma cells. Table 3 shows that increased phagocytosis occurred when macrophages only were treated with the enzyme. Treatment of both cell types did not increase the degree of ingestion above that when either macrophages or lymphoma cells were separately treated. Thus, whatever the mechanism of increased uptake following neuraminidase-treatment of cells may be, the mode of action was obviously not specifically directed towards L5178Y cells.

(b) *Trypsin treatment.* Table 4 summarizes the effect of different concentrations of trypsin on L5178Y cells prior to opsonization with antiserum. It is seen that phagocytosis was facilitated and that the optimal concentration of trypsin to produce this effect was 0·25–0·5 per cent.

To ascertain if trypsin treatment of macrophages also resulted in increased phagocytosis, slip cultures were incubated with 0·25 per cent trypsin for 30 minutes, followed by washing and incubation with 5×10^5 untreated and trypsin-treated lymphoma cells suspended in serum diluted to 1/50. Approximately 80 per cent of the trypsin-treated macrophages ingested one to eight lymphoma cells per macrophage compared with 50–65 per cent of untreated macrophages which contained up to two lymphoma cells per macrophage.

TABLE 4

EFFECT OF TRYPSIN-TREATMENT OF L5178Y CELLS ON PHAGOCYTOSIS* BY NORMAL MACROPHAGES

L5178Y cells incubated with:	Per cent macrophages† with ingested L5178Y cells
Fischer's medium only	50
0·1 per cent trypsin	65
0·25 per cent trypsin	80
0·50 per cent trypsin	80
1·0 per cent trypsin	70

* Normal and anti-L5178Y serum diluted to 1/50.
† Mean of two slip cultures per dilution.

Trypsin treatment of both macrophages and lymphoma cells slightly improved the degree of phagocytosis above the level found when macrophages only were treated.

DISCUSSION

The above data and those reported by others (Bennett *et al.*, 1963, 1964) indicate that a variety of exogenous factors may affect *in vitro* phagocytosis of tumour cells. The finding that macrophages from mice injected with thioglycollate ingested more L5178Y cells than those from normal mice is in agreement with that of Bennett *et al.* (1963) who used macrophages from mice injected with starch. As yet the mechanism of increased uptake of tumour cells has not been elucidated, though it is likely that the increased volume of cytoplasm and rapid spreading ability play a vital role (North, 1968).

Phagocytosis of lymphoma cells by normal macrophages was shown to be related to the dilution of antiserum employed and to the number of L5178Y cells added to each macrophage slip culture. Inocula of 10^5 and 10^6 L5178Y cells were able to detect opsonic activity up to a serum dilution of 1/1250, while those of 10^3 and 10^4 were unable to do so beyond 1/250. These results suggest that the frequency of cell–opsonin interaction decreases more rapidly with lower cell inocula than with higher inocula.

The effect of enzymic treatment of cells on the degree of phagocytosis was striking. The increased uptake of lymphoma cells following trypsin or neuraminidase treatment of either L5178Y cells or macrophages suggests that opsonic factors of primary immune serum may bind more readily or in greater quantities to the treated than to the untreated cell types. Moreover, the mechanism of action may be similar for both types in that enzyme-treatment may 'unmask' antigenic sites, as suggested by Burnet and Anderson (1947) and Currie and Bagshawe (1968). However, since previous results showed that only a small proportion of total opsonic activity of primary immune serum was due to specific cytotoxic antibody, and that the major fraction was non-cytotoxic with a low avidity for L5178Y cells (Evans, 1971), a hypothesis based on increased binding must involve the non-cytotoxic γ-globulin of primary immune serum.

The relevance of this opsonic test to the *in vivo* rejection mechanism of L5178Y cells by C57Bl mice is not clear. At present, we do not know whether the opsonic activity detected *in vitro* plays the same role *in vivo*, nor is it established that phagocytosis of intact L5178Y cells is an essential step for the initiation of the immune response, as may be the case for some other types of tumours (Amos, 1960; Journey and Amos, 1962; Yamada, Yamada and Hollander, 1969). Preliminary evidence from this laboratory suggests that little or no phagocytosis of intact lymphoma cells takes place in the peritoneal cavity, the main reaction of cell killing appearing to occur by cell contact. A similar type of reaction was reported by Journey and Amos (1962) during rejection of a C3H ascitic sarcoma. It is quite possible that *in vivo* opsonic factors may facilitate contact between the macrophages and the target lymphoma cells, or that they may assist in phagocytosis of broken-down lymphoma cells. The appearance of opsonic factors for L5178Y cells at approximately the same time as the appearance of cytotoxic antibody (Evans, 1971) may provide the basis for a general *in vitro* model system to detect changes in an immunized animal.

ACKNOWLEDGMENTS

This investigation was supported by grants from the Medical Research Council and the British Empire Cancer Campaign.

The author would like to thank Professor P. Alexander for his criticism and advice.

REFERENCES

AMOS, D. B. (1960). 'Possible relationship between the cytotoxic effects of isoantibody and host cell function' *Ann. N.Y. Acad. Sci.*, **87**, 273.

BENNETT, B., OLD, L. J. and BOYSE, E. A. (1963). 'Opsonization of cells by isoantibody *in vitro*.' *Nature (Lond.)*, **198**, 10.

BENNETT, B., OLD, L. J. and BOYSE, E. A. (1964). 'Peritoneal macrophages as immunologically active cells in tissue rejection.' In: *Retention of Functional Differentiation in Cultured Cells. Wistar Inst. Symp. Monogr.* **1**, 87.

BURNET, F. M. and ANDERSON, S. G. (1947). 'The "T" antigen of guinea-pig and human red cells.' *Aust. J. exp. Biol. med. Sci.*, **25**, 213.

CURRIE, G. A. and BAGSHAWE, K. D. (1968). 'The effect of neuraminidase on the immunogenicity of the Landschutz ascites tumour: site and mode of action.' *Brit. J. Cancer*, **22**, 588.

EVANS, R. (1971) 'Phagocytosis of Murine Lymphoma Cells by Macrophages. II. Differences between opsonic and cytotoxic activity in serum allogeneic to a murine lymphoma.' *Immunology*, **20**, 75.

FISCHER, G. A. (1958). 'Studies of the culture of leukaemia cells *in vitro*.' *Ann. N.Y. Acad. Sci.*, **67**, 673.

JOURNEY, L. J. and AMOS, D. B. (1962). 'An electron microscope study of histiocyte response to ascites tumour homografts.' *Cancer Res.* **22**, 998.

NORTH, R. J. (1968). 'The uptake of particulate antigens.' *J. Reticulo-endoth. Soc.* **5**, 203.

YAMADA, H., YAMADA, A. and HOLLANDER, V. P. (1969). 'Role of cellular and humoral factors in the destruction of nascent plasma cell tumours.' *Cancer Res.* **29**, 1420.